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Effects and Ultrastructural Localization of
IgG and IgA Antibodies on Eimeria falciformis

by

William Michael Whitmire

B.A., University of Montana, 1974

B.S., University of Montana, 1978

Presented in Partial Fulfillment of the
Requirements for the Degree of

Master of Science

UNIVERSITY OF MONTANA

1984

Approved by:

CASpeer
Chairman, Board of Examiners

RC Murray
Dean, Graduate School

12/27/1984
Date

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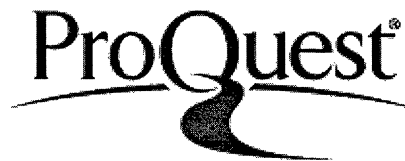
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Whitmire, William M., MS December 1984

Microbiology

Effects and Ultrastructural Localization of IgG and IgA
Antibodies on Eimeria falciformis

Director: C.A. Speer *CAS*

The localization and effects of parasite-specific IgG and IgA immunoglobulins on Eimeria falciformis sporozoites and merozoites were examined by immunoelectron microscopy. Purified specimens were fixed in glutaraldehyde, incubated with heat-inactivated sera or cecal contents from normal or specifically-immunized mice, reacted with ferritin- or colloidal gold-conjugated sheep or goat antimouse IgG or IgA antibody and prepared for transmission electron microscopy. Other purified samples of sporozoites and merozoites were exposed to sera or cecal contents for 5, 10, 15, and 30 minute intervals, fixed in 0.15% glutaraldehyde, and then incubated with ferritin- or colloidal gold-conjugated sheep or goat antimouse antibody.

Parasite-specific IgG and IgA receptor sites were detected on the plasmalemma of sporozoites and merozoites. Specific IgG immunodeterminate sites were also present on the inner and outer layers of the oocyst wall, and on the inner surface of the sporocyst wall. Live sporozoites and merozoites shed immune complexes at their posterior ends. This phenomenon may be a defense mechanism which enables the parasite to evade destruction by the host humoral response.

No internal alterations were detected ultrastructurally in sporozoites or merozoites treated with parasite-specific IgG or IgA antibodies.

In order to determine the effect of parasite-specific IgG on parasite development in vivo, sporozoites and merozoites were incubated with heat-inactivated sera from normal or specifically-immunized mice and then injected into the ceca of normal mice. A 64.5% and 63.2% decrease in oocyst-output occurred in mice injected intracecally with sporozoites or merozoites, respectively, that had been treated previously with antisera from immunized mice. Thus, parasite-specific antisera provided only partial protection against the ability of sporozoites and merozoites to undergo development to oocysts in vivo.

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ABBREVIATIONS

In Text

DAI	days after inoculation
FA	fluorescent antibody
Fe-IgG	ferritin-conjugated goat antimouse IgG
xg	gravity
gm	gram
HBSS	calcium and magnesium deficient Hank's balanced salt solution
hr	hour
IEM	immunolectron microscopy
IFA	indirect fluorescent antibody
I.P.	intraperitoneal
Kg	kilogram
M	molar
min	minute
ml	milliliter
mg	milligram
mM	millimolar
mm	millimeter
MPB	Millonig's phosphate buffer
nm	nanometer
PBS	phosphate buffered saline
rpm	revolutions per minute
RT	room temperature
sec	second

TEM	transmission electron microscopy
UV	ultraviolet
v/v	volume to volume
w/v	weight to volume

In Figures

Am	amylopectin
Ar	anterior refractile body
Cg	colloidal gold
Co	conoid
Fe	ferritin
Fp	flagellated protozoan
Go	Golgi apparatus
Im	inner membrane complex
Iw	inner layer of oocyst wall
Mi	mitochondrion
Mn	microneme
Mt	microtubule
Nu	nucleus
Ow	outer layer of oocyst wall
Pf	pellicular fold
Pl	plasmalemma
Pr	posterior refractile body
Rh	rhoptry
Sb	Stieda body
Sp	sporozoite

Sw	sporocyst wall
Vs	vesicle

INTRODUCTION

Taxonomic Classification of Eimeria falciformis

Subkingdom: PROTOZOA (Goldfuss 1818, emend. Siebold 1845)

Phylum: APICOMPLEXA (Levine 1970)

Class: SPOROZOEAE (Leuckart 1879)

Subclass: COCCIDIA (Leuckart 1879)

Order: EUCOCCIDIIDA (Leger and Duboscq 1910)

Suborder: EIMERIINA (Leger 1911)

Family: EIMERIIDAE (Minchin 1903)

Genus: EIMERIA (Schneider 1875)

Species: FALCIFORMIS (Schneider 1875)

History. In a historical review of the coccidia, Levine (1973) states that in 1674 Leeuwenhoek saw oocysts of Eimeria stiedai in the bile ducts of a rabbit (29). This was the first protozoan ever to be seen. It was not until 1839, more than 150 years later, that Hake further described this parasite in which he thought that the oocysts were pus globules originating from liver carcinoma in rabbits. Then in 1847, Kauffman described sporulation within the oocyst, followed by the delineation of the endogenous life cycle by Eimer in 1870. Eimer (1870) then gave a brief description of the life cycle of "Gregarina falciformis" in mice. This organism was later renamed Eimeria falciformis by Schneider (1875) and became the type species of the genus Eimeria (29). The entire

life cycle of Eimeria falciformis was described by Schuberg in 1895. Presently, the genus Eimeria, contains over a thousand species which occur mainly in vertebrate hosts (42).

General. Coccidiosis is an acute intestinal disease that occurs in various species of animals, including chickens, turkeys, rabbits, sheep and cattle. This disease is essentially a disease which results from abnormal crowding of a host species in a limited area (29). Etiologic agents of coccidiosis are members of the genus Eimeria. Most Eimeria species are obligate intracellular monoxenous parasites that have a high degree of host specificity (27, 36). They generally infect digestive tract tissues and cause diarrhea, destruction of intestinal epithelium, weakness, weight loss, retardation of growth and death (33,39). In underdeveloped countries with low food production, the losses incurred by these parasites may be devastating.

Fitzgerald (1972) estimated that bovine coccidiosis resulted in a global monetary loss of 472 million dollars (19). Other investigators have determined that in the United States alone, 60 to 120 million dollars are lost each year by the poultry industry (57, 59). This sum does not include the cost of anticoccidial drugs which amount to another 35 million dollars per annum (59). Without question, these parasites are entities of considerable economic impact throughout the world. To date, there is no vaccination in existence nor any satisfactory preventative measures pertaining

to infections with these parasites (18). Hopefully some sort of prophylactic treatment will be developed in the near future.

Life cycle. The typical life cycle of an eimerian includes endogenous stages inside the host as well as exogenous stages occurring outside of the host. Eimeria species have oocysts that contain four sporocysts each containing two sporozoites (29). Infection begins with the ingestion of sporulated oocysts by a suitable species of host. Upon exposure to carbon dioxide, trypsin and bile in the intestinal tract, sporozoites excyst from oocysts and penetrate the intestinal epithelium (34, 60). Sporozoites of E. falciiformis penetrate the epithelium of the cecum and large intestine. Intracellular sporozoites undergo asexual reproduction by a process called merogony (schizogony) to form merozoites which escape from the host cell into the lumen of the large intestine and then penetrate other epithelial cells. This process is repeated 2 or more times. Finally, merozoites of the last generation escape from host cells, invade adjacent or other epithelial cells and develop into micro- or macrogamonts. Microgamonts escape from their host cells and fertilize macrogamonts. The resulting zygotes develop into oocysts which are shed into the feces (23, 31). Once outside of the host, oocysts undergo sporulation upon exposure to oxygen to become infective to another suitable host (60).

Immunity. Little is known about the immune mechanisms involved in Eimeria infections. However, infections by Eimeria are species-specific and cause some degree of resistance to reinfection (6, 44-45, 63). Both antibody and cell-mediated mechanisms have been implicated in acquired immunity to coccidiosis, but their respective roles have not been well defined (22, 37, 48, 50, 53, 55-56, 58). Furthermore, specific Eimeria immunogens for eliciting protective immunity have not been identified (63). IgA antibodies may be involved in the immune response to coccidia because of their secretory nature and their ability to withstand exposure to proteolytic enzymes (5, 11-14, 56, 69). In general, the secretory immune system appears to function by immobilizing microorganisms or antigens on mucosal surfaces, thus impeding their entry into host tissues (56, 58). If such a mechanism is operative in immune responses to Eimeria species, it is probably directed against sporozoites and merozoites (48, 58, 68). Sporozoites and merozoites are motile stages and are occasionally extracellular and must penetrate intestinal epithelial cells in order to undergo further asexual or sexual development.

Immunofluorescent antibody and precipitation assays have shown that a systemic IgG immunoglobulin response occurs during Eimeria infections (11, 26, 35, 44, 66). However it is debatable whether or not IgG antibodies contribute to protection against Eimeria species because they are not normally

found in body secretions (13). Yet, the possibility of increased vascular permeability resulting from the inflammatory process which is elicited by the disease, may allow interaction between IgG and the parasite (52).

Immunoelectron microscopy. The techniques of immunoelectron microscopy (IEM) have proven to be valuable in elucidating specific antigenic determinate sites. For example, recent IEM work by electron microscopists have revealed intimate host-parasite immunogenic relationships concerning Toxoplasma gondii and Legionella pneumophila, and other workers have capitalized on these techniques to visualize the exact location of cell components and surface antigens as well (20, 24, 38, 43, 61, 63, 70).

One of the most popular markers used for ultrastructural visualization of immunogenic sites is an antibody conjugated to either ferritin or colloidal gold (1, 24, 70). Ferritin and colloidal gold can be recognized by transmission electron microscopy (TEM) because both possess characteristic electron opacity and structural shape. Ferritin is a spherical molecule with a diameter of 12 nanometers (nm), a molecular weight of 650,000 and an iron content of 23% by weight. This iron is concentrated into characteristic micelles in the center of the molecule, forming a tetrad with a diameter of 5 nm. Colloidal gold particles have a homogeneous particle size of 10-40 nm, depending on the manufacturer.

Except for the work of Speer et al. (62-64) concerning monoclonal antibodies, there have been no previous IEM investigations using immunodeterminate site markers on Eimeria species.

Objectives. The objectives of this investigation were:

- a.) to study the localization of IgG and IgA receptor sites on the plasmalemma of sporozoites and merozoites of E. falciformis.
- b.) to determine the effects of IgG and IgA antibodies on sporozoites and merozoites of E. falciformis.

Rationale. The use of ferritin and colloidal gold conjugated antibodies with TEM will allow for the precise determination of IgG and IgA specificity as well as any mobilization of immune complexes on the surface of sporozoites and merozoites. In addition, any alteration that might occur to the parasite as a result of exposure to these antibodies might be visible at the ultrastructural level.

The effects of parasite-specific immunoglobulin on parasite development will be determined by utilizing an in vivo system. The numbers of oocysts produced in the feces of non-immune animals inoculated intracecally with sporozoites or merozoites that were previously exposed to parasite-specific immunoglobulins should give an indication of their effects upon parasite development.

MATERIALS AND METHODS

Experimental animals. Ten to 13 week-old BALB/c laboratory mice were obtained from a breeding colony at the University of Montana (Missoula, Montana). These mice were coccidia-free and were placed in strict isolation following inoculation with Eimeria falciformis. Feed and water were given to mice when necessary except in the case of impending surgical procedures in which animals were starved for 12 hours before surgery.

Parasite. The strain of Eimeria falciformis that was used throughout the course of experimentation was originally obtained from John V. Ernst, Animal Parasitology Institute, U.S. Department of Agriculture, Beltsville, Maryland 20705. Maintenance of the parasite was achieved by serial passage in laboratory mice.

Production, collection and storage of oocysts. At 7 to 10 days after an initial per os inoculation of 10^3 sporulated oocysts of E. falciformis, infected mice were placed in wire bottom cages that were situated over trays containing water. Fecal droppings produced from these mice were collected daily from the water. Oocysts were concentrated, sporulated in a 2.5% (w/v) $K_2Cr_2O_7$ solution in distilled water and cleaned by the methods described by Davis (9). Sporulated oocysts were then pooled and stored at $4^{\circ}C$ in distilled water.

Immune sera. Mice inoculated initially with 10^3 sporulated oocysts were challenged with 10^3 oocysts on days 21 through 25 days after inoculation (DAI). Three days later they were anaesthetized with ether and exsanguinated. The blood was pooled, allowed to clot on ice and then centrifuged at 2000 rpm for 10 min to separate the serum from the red blood cells. Serum was removed, heat-inactivated at 56°C for 30 min, aliquoted into 1 ml samples and stored at -20°C . This serum pool is referred to as immune serum and is predominantly of the IgG immunoglobulin fraction.

Normal sera. Coccidia-free mice were bled in the same manner as the immunized animals and the serum was separated from other blood elements, heat-inactivated and aliquoted in 1 ml samples. This serum was stored at -20°C until used and is referred to as normal serum.

Immune cecal contents. Immunized mice (see above) were sacrificed by cervical dislocation and their ceca and colons were removed immediately. The colon and cecum of each mouse was cut longitudinally and their contents were collected in 0.5 ml cold phosphate buffered saline (PBS) at pH 7.2. The mucosa of the cecum and upper one-third of the colon was removed by gently scraping with a microscope slide and added to the suspension of cecal and colonic contents. The suspension was mixed with a Vortex mixer for 5 min and subjected to freeze-thawing (three times). Samples were heated just long enough to melt all of the ice in order to minimize

proteolysis (11). After centrifuging the samples at 1000 xg for 10 min, the supernatant was removed, aliquoted into 2 ml units and stored at -20°C . This material is referred to as immune cecal contents, which contained specific IgA immunoglobulins.

Normal cecal contents. Animals that were coccidia-free and had not been previously exposed to coccidia were killed and their colons and ceca were removed and processed as described for immune animals. This preparation is referred to as normal cecal contents.

Conjugates. In order to detect parasite-specific immunoglobulins, fluorescein-conjugated sheep antimouse IgG (heavy and light chain) and goat antimouse IgA (alpha chain), and ferritin-conjugated goat antimouse IgG (heavy and light chain) (United States Biochemical Corporation, Cleveland, Ohio) were used for light and transmission electron microscopy (TEM), respectively. Colloidal gold (10 nm particle size)-conjugated goat antimouse IgA (alpha chain) (E Y Laboratories, Inc., San Mateo, California) was also used to detect specific IgA by TEM. All conjugated immunoglobulins were diluted 1:20 with PBS before use.

Preparation of sporozoite antigen. Clean sporulated oocysts were suspended in calcium and magnesium deficient Hank's balanced salt solution (HBSS) (40) at pH 7.4 and broken by grinding with a motor-driven teflon coated tissue grinder. When most of the sporocysts were released from

the oocysts, an equal volume of excysting fluid (0.5 gm trypsin, 1.5 gm Na taurocholate in 100 ml HBSS, pH 7.7) was added to the suspension, which was then incubated at 37°C for 1 to 1½ hours. Free sporozoites were washed clean of excystation fluid by centrifuging and resuspending them in HBSS. In order to separate sporozoites from sporocysts, oocysts and oocyst walls, the suspension was passed through nylon wool (Leuco-pak, Fenal Laboratories, Deerfield, Illinois) as described (28). The eluate contained mostly sporozoites, and a few oocysts, oocyst walls and sporocysts.

Preparation of merozoite antigen. BALB/c mice were sacrificed at 5 DAI of each mouse with 2×10^4 sporulated oocysts. The cecum and colon were removed from each animal, cut longitudinally, and processed as described previously (4, 28) in order to isolate third-generation merozoites. Briefly, luminal contents were removed from the cecum and colon by three 15 sec vigorous washes in 50 ml HBSS. Tissues were then placed in 50 ml HBSS containing 1 mM dithiothreitol (Sigma, St. Louis, Missouri) and stirred with a magnetic stirrer for 1 min at room temperature (RT). Tissues were then cut into several half-inch pieces, placed in 50 ml HBSS containing 0.7 mM EDTA (Sigma, St. Louis, Missouri) and stirred by a magnetic stirrer for 1 to 1½ hours at RT. The supernatant was passed through a fine nylon mesh, centrifuged at 200 xg for 10 min and resuspended in 20 ml HBSS. Merozoites were purified by passing the suspension through

a nylon wool column and collecting the eluate (28).

Preparation of fluorescent antibody slides. Concentrated suspensions (0.05 ml) of purified sporozoites or merozoites of E. falciformis were placed on multi-welled Toxoplasma microscope slides, dried, fixed in acetone, and stored at -20°C in plastic slide boxes.

Indirect immunofluorescence procedure. The indirect fluorescent antibody (IFA) technique used here was similar to the procedure described by Hudson and Hay (25). Sera and cecal contents from immunized and non-immunized animals were thawed, diluted 1:5 to 1:160 with PBS, applied to the multi-welled Toxoplasma slides containing sporozoites or merozoites of E. falciformis and incubated for 30-45 min at RT. Specimens on slides were then washed in PBS, incubated with fluorescein-conjugated antimouse globulins for 30 min at RT, washed twice in PBS, once in distilled water and air dried. Three drops of FA mounting fluid (Difco Laboratories, Detroit, Michigan) were added to each slide followed by application of a glass coverslip. The slides were then examined with a model 18 Zeiss UV light microscope. Degree of fluorescence was rated on a scale of - to 4+. Tests of fluorescence for each dilution were performed two or more times. Information gained from these IFA tests were used to determine the appropriate titers of immune sera and immune cecal contents for preparing specimens for immunoelectron microscopy.

Immunoelectron microscopy. Sporozoites (7×10^6) or merozoites (1.5×10^7) were fixed in 0.15% (v/v) glutaraldehyde in Millonig's phosphate buffer (MPB) pH 7.4 for 20 min at RT, washed twice with HBSS, centrifuged and then reacted with 1:20 dilutions of immune sera or normal sera in 0.15 M PBS (pH 7.2), or 1:5 dilutions of immune or normal cecal contents in PBS for 30-45 min at RT. Parasites were washed twice with PBS and then incubated at RT for 30 min with 0.1 ml ferritin- or colloidal gold-conjugated antimouse globulins that had been previously diluted to 1:20 with 0.05 M PBS (pH 7.2). Samples were then washed twice in PBS and centrifuged at 1000 xg for 5 min. Pellets were fixed with 2.5% (v/v) glutaraldehyde in MPB, postfixed in 1% (w/v) OsO_4 , dehydrated in ethanol, and embedded in Spurr's medium. Thin sections were cut with a Sorvall MT 5000 (DuPont, Wilmington, Delaware), stained with uranyl acetate and lead citrate, and examined by a Zeiss EM9-S2 or a JEOL 100 CX transmission electron microscope.

In order to determine if sporozoites or merozoites were capable of capping immune complexes on their surfaces, parasites were prepared as follows. Sporozoites or merozoites were exposed to sera or cecal contents for 5, 10, 15, or 30 min at RT, fixed with 0.15% (v/v) glutaraldehyde, washed twice with PBS and then prepared for TEM as above.

In vivo inoculations of sporozoites and merozoites. Forty-eight female BALB/c mice that were 12-13 weeks old,

weighed approximately 25 gm each, and were coccidia-free, were placed in six groups, each with eight animals. The animals were starved for 12 hr, anaesthetized by intraperitoneal (I.P.) inoculation of 0.05 ml sodium pentobarbitol (40 mg/Kg body weight) and incised longitudinally along the abdomen to expose the cecum. Mice in each group were inoculated intracecally with 0.5 ml volumes containing 6×10^4 sporozoites or 1.25×10^5 merozoites that had been previously treated for 15 min at RT with HBSS or a 1:5 dilution of heat-inactivated immune or normal serum in PBS. After inoculation, the cecum was returned to the peritoneal cavity and the incision closed by metal surgical clips and a commercial superglue. After recovering from the anaesthesia, each group of surviving mice (at least 5 per group) was placed in a clean cage and all the feces were collected during patency which occurred at days 6-8 and 2-4 after inoculation of sporozoites and merozoites, respectively. Oocysts from each group were isolated, counted in quadruplicate by a hemocytometer, averaged and recorded (Table I). After patency, mice were sacrificed and autopsied for evidence of post-surgical lesions or complications that might have hindered parasite development or oocyst passage. None were found in any of the animals.

RESULTS

Effects of parasite-specific IgG on parasite development in vivo. Tables I and II show the relative numbers of oocysts produced by BALB/c mice that were inoculated intracably with sporozoites or merozoites that had been previously incubated with HBSS, heat-inactivated immune serum or normal serum. Table I displays the average daily and total number of oocysts produced by each animal within their respective groups over a 3 day patency period. Table II shows total oocyst-output, expressed as a percentage of values obtained from HBSS-treated groups. Treatment of sporozoites and merozoites with immune serum resulted in a significant decrease in oocyst-output compared to those organisms which were exposed to HBSS or normal serum. Treatment of sporozoites or merozoites with normal serum resulted in a 19.4% and 18.4% reduction in oocyst-output, whereas immune serum caused a 64.5% and 63.2% reduction in oocyst-output, respectively (Table II).

Indirect immunofluorescence. The indirect immunofluorescence antibody test (IFA) showed that the IgG immunoglobulin fraction of immune serum obtained from BALB/c mice reacted specifically against both sporozoites and merozoites (Figs. 1,2). Sporozoites and merozoites exhibited strong fluorescence (3+ to 4+) upon exposure to 1:5 to 1:160 dilutions of immune serum whereas parasites exposed to normal

serum were negative (Fig. 4).

Variable IFA results were obtained with parasites exposed to parasite-specific IgA in immune cecal contents. The strongest IFA reaction observed was considered to be 1+ (Fig. 3). All other attempts to detect parasite-specific IgA resulted in weak or negative IFA reactions. Cecal contents from normal animals and serum from immune animals also gave negative fluorescence for IgA.

Based on the above information, normal or immune serum was diluted 1:20 (unless stated otherwise) and normal or immune cecal contents were diluted 1:5 for immunoelectron microscopy (IEM) techniques.

Immunoelectron microscopy. Sporozoite preparations contained oocysts, oocyst walls and excysted sporocysts in addition to sporozoites. The oocyst wall was 312 nm thick and had two layers, an inner electron-lucent layer and an electron-dense outer layer (Fig. 7). Both layers completely surrounded the oocyst; the inner layer was uniform in thickness (212 nm; range, 180-240 nm; n=15), whereas the outer layer was variable in thickness (100 nm; 25-150 nm; n=15) being thinner at the micropyle (Fig. 8). Two closely applied membranes were interposed between the outer and inner layers. Usually, only the inner layer of the oocyst wall was present because treatment with Clorox had removed the outer layer (Figs. 5, 6). In oocysts in which the outer layer was missing, one or both of the 'interposed' membranes

were still attached to the outer surface of the inner layer (Fig. 9).

Sporocysts were surrounded by a sporocyst wall that consisted of two closely applied layers, an outer electron-lucent layer and an inner layer which was a unit membrane (Fig. 6). Stieda and substieda bodies were located at one pole of the sporocyst. Each sporocyst contained two sporozoites and a sporocyst residuum that consisted of amylopectin granules, lipid droplets, membrane-bound vesicles and granular material (Fig. 10).

Sporozoites had all of the organelles characteristic of the Apicomplexa and Eimeria species such as a single nucleus, refractile bodies, conoid, polar and apical rings, micronemes, rhoptries, subpellicular microtubules, mitochondria, Golgi, smooth and rough endoplasmic reticulum, ribosomes, amylopectin granules and lipid droplets (Fig. 11) (41). Third-generation merozoites had a similar ultrastructure except that they contained no refractile bodies (Fig. 12).

Parasites prefixed in 0.15% glutaraldehyde, exposed to serum or cecal contents from immune mice and then ferritin- or colloidal gold-conjugated antibodies exhibited characteristic ferritin or gold binding patterns.

Ferritin-conjugated antimouse IgG attached to the outer surface of intact oocysts but was not present on interior structures, indicating that parasite-specific IgG and ferritin-conjugated antibody did not enter intact oocysts (Fig. 5).

Parasite-specific IgG and ferritin-conjugated antibodies entered fractured oocysts and reacted specifically with various structures. Ferritin localized in patches or as a uniform layer on the inner and outer layers of the oocyst wall (Figs. 5-9). In those specimens in which both layers of the oocyst wall were intact, ferritin attached to the outer surface of the outer layer and to the inner surface of the inner layer (Figs. 7, 8). In those in which the outer layer had been removed by Clorox, ferritin attached to the exposed outer surface of the inner layer, which was usually covered by one or both of the 'interposed' membranes (Fig. 9).

Ferritin did not attach to intact sporocysts. However, in sporocysts in which the Stieda body had undergone dissolution in the presence of excysting fluid (trypsin and bile salt), ferritin was localized in patches on the inner surface of the sporocyst wall, on lipid droplets of the sporocyst residuum and on the plasmalemma of sporozoites (Fig. 10). No ferritin attached to amylopectin granules located free within sporocysts. Relatively more ferritin attached to the inner surface of the sporocyst wall than to sporozoites within sporocysts. Free sporozoites had relatively more ferritin on their surfaces than those still within sporocysts (compare Figs. 10 and 11). Ferritin was localized in patches that were uniformly distributed over the sporozoite plasmalemma (Fig. 11). Relatively more ferritin attached to the

surface of merozoites than sporozoites (compare Fig. 11 with 12, 14-16). . Occasionally, membrane-bound vesicles labeled with ferritin were found in close proximity to the surface of prefixed merozoites (Fig. 16). Such vesicles were never observed in prefixed sporozoite preparations.

Moderate amounts of ferritin were found on sporozoites or merozoites exposed to immune serum for 5 min and then fixed in glutaraldehyde before treatment with Fe-IgG. However, in preparations postfixed at 10, 15, and 30 min after similar treatment, little or no ferritin was found on their surfaces (Fig. 18). Occasionally, ferritin-labeled aggregates were observed near or at the posterior ends of merozoites after a reaction interval of 10 min (Fig. 17). In preparations postfixed at 10, 15, and 30 min, ferritin was found attached to large vesicles and granular material (Fig. 18). Ferritin-labeled aggregates were observed in post-fixed sporozoite preparations, but never in association with the surface of sporozoites.

In IgA prefixed preparations exposed to immune cecal contents and colloidal gold-conjugated goat antimouse IgA, no gold label was found on oocysts, oocyst walls or sporocysts and relatively little label was found on the surfaces of sporozoites and merozoites (Figs. 20, 21). Capping or shedding of immune complexes involving IgA on post-fixed sporozoites and merozoites was not observed.

No internal ultrastructural alterations were detected

in sporozoites or merozoites exposed to immune serum or immune cecal contents. Parasites exposed to HBSS, serum or cecal contents from normal mice and then ferritin- or gold-conjugated goat antimouse IgG or IgA had no ferritin or gold attached to their surfaces (Figs. 13, 19).

Some of the merozoite preparations contained a few flagellated protozoans such as Trichomonas and Hexamita species (Fig. 12). Ferritin or gold was never found on their surfaces.

Table I. Oocyst-output by BALB/c mice inoculated previously with E. falciformis sporozoites or merozoites treated with HBSS, normal, or immune serum.

Stage of parasite	Inoculum per mouse	Treatment	Dilution	Oocyst-output/mouse/day of patency ¹			
				Day 1	Day 2	Day 3	Total
Sporozoite	6.0×10^4	HBSS	0	6.0×10^3	1.1×10^6	2.0×10^6	3.1×10^6
		Normal serum	1:5	9.3×10^3	1.4×10^6	1.1×10^6	2.5×10^6
		Immune serum	1:5	1.0×10^3	3.0×10^5	7.5×10^5	1.1×10^6
Merozoite	1.25×10^5	HBSS	0	9.5×10^3	2.6×10^4	2.3×10^3	3.8×10^4
		Normal serum	1:5	7.8×10^3	2.2×10^4	1.6×10^3	3.1×10^4
		Immune serum	1:5	9.0×10^2	1.2×10^4	1.2×10^3	1.4×10^4

¹Oocysts were passed at 6-8 days and 2-4 days after intracecal inoculation of sporozoites or merozoites, respectively.

Table II. Percent decrease in oocyst-output by BALB/c mice inoculated intracecally with normal or immune serum treated E. falciformis sporozoites or merozoites as compared to HBSS-treated controls.

Stage of parasite	Treatment	Percent decrease in oocyst-output ¹
	Normal serum	19.4
	Immune serum	64.5
	Normal serum	18.4
	Immune serum	63.2

¹Percent decrease in oocyst-output was determined for the total number of oocysts produced during days 6-8 and days 2-4 post-inoculation of sporozoites or merozoites, respectively.

EXPLANATION OF ILLUSTRATIONS

Plate 1. E. falciformis sporozoites and merozoites: Examples of the reactions obtained by the indirect fluorescent antibody test.

Figure 1. Sporozoites exhibiting a 4+ reaction. Treatment: Prefixed, immune serum, fluorescein-conjugated sheep antimouse IgG. X 750.

Figure 2. Merozoites exhibiting a 3+ reaction. Treatment: Prefixed, immune serum, fluorescein-conjugated sheep antimouse IgG. X 750.

Figure 3. Merozoites exhibiting a 1+ reaction. Treatment: Prefixed, immune cecal contents, fluorescein-conjugated goat antimouse IgA. X 450.

Figure 4. Merozoites exhibiting a negative reaction. Treatment: Prefixed, normal serum, fluorescein-conjugated sheep antimouse IgG. X 450.

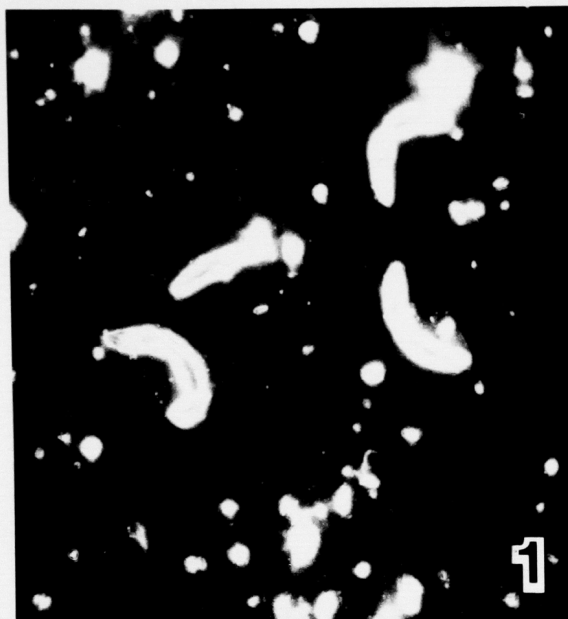


Plate 2. Ultrastructural localization of specific IgG immunoglobulins and ferritin-conjugated goat antimouse IgG on the surface of an intact oocyst.

Figure 5. Low magnification of intact oocyst with ferritin (Fe) attached on the outer margin of the exposed inner wall (Iw). Sb, Stieda body; Sp, sporozoite. Treatment: Prefixed, immune serum, ferritin-conjugated goat antimouse IgG. X 9,900.



Plate 3. Ultrastructural localization of parasite-specific mouse IgG by ferritin-conjugated goat antimouse IgG on layers of the oocyst wall.

Figure 6. Higher magnification of portion of oocyst in Figure 5. Note ferritin (Fe) on the outer surface of the inner layer (Iw) of oocyst wall. Sb, Steida body; Sw, sporocyst wall. Treatment: Prefixed, immune serum, ferritin-conjugated goat antimouse IgG. X 60,000.

Figure 7. High magnification showing inner (Iw) and outer (Ow) layers of the oocyst wall. Note that ferritin (Fe) is localized in clumps on the outer and inner layers, respectively. Treatment: Prefixed, immune serum, ferritin-conjugated goat antimouse IgG. X 99,000.

Figure 8. Intermediate magnification of oocyst wall near micropyle. Treatment: Prefixed, immune serum, ferritin-conjugated goat antimouse IgG. X 60,000.

Figure 9. Inner layer of the oocyst wall with ferritin on both surfaces. The 'interposed' membranes identify the outer surface. Treatment: Prefixed, immune serum, ferritin-conjugated goat antimouse IgG. X 78,000.

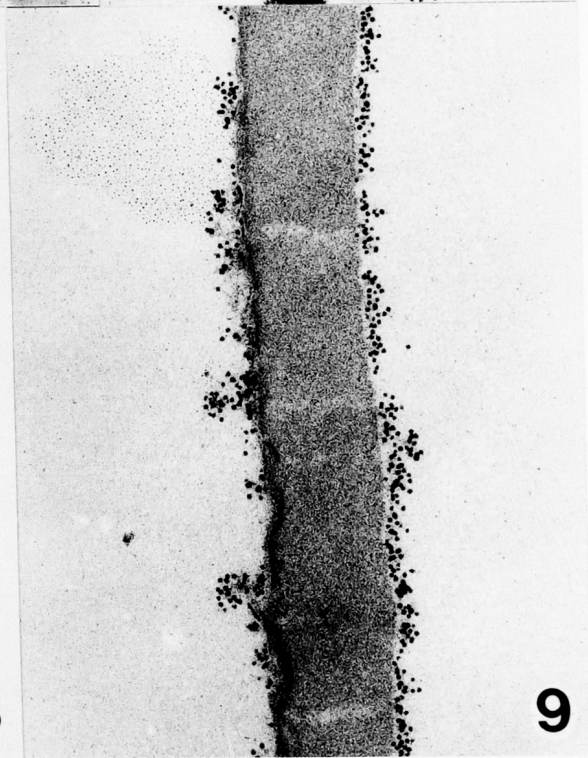
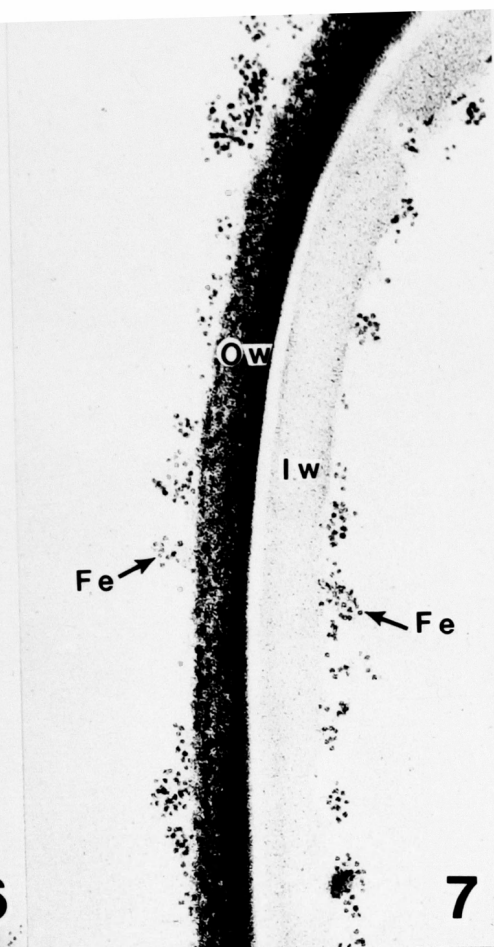
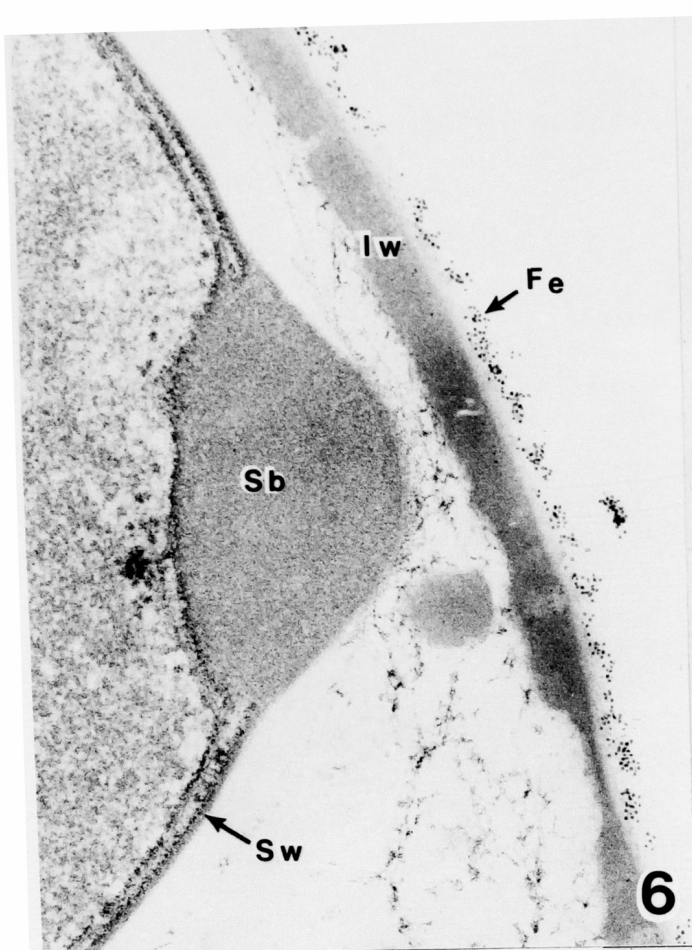


Plate 4. Sporocyst with ferritin on the inner surface of sporocyst wall.

Figure 10. Sporocyst with two sporozoites; note that relatively more ferritin (Fe) has attached to the inner surface of the sporocyst wall (Sw) than to the sporozoite plasmalemma. Am, amylopectin; Ar, anterior refractile body; Co, conoid; Go, Golgi apparatus; Mi, mitochondrion; Mn, micronemes; Nu, nucleus; Pr, posterior refractile body. Treatment: Prefixed, immune serum, ferritin-conjugated goat antimouse IgG. X 19,000.

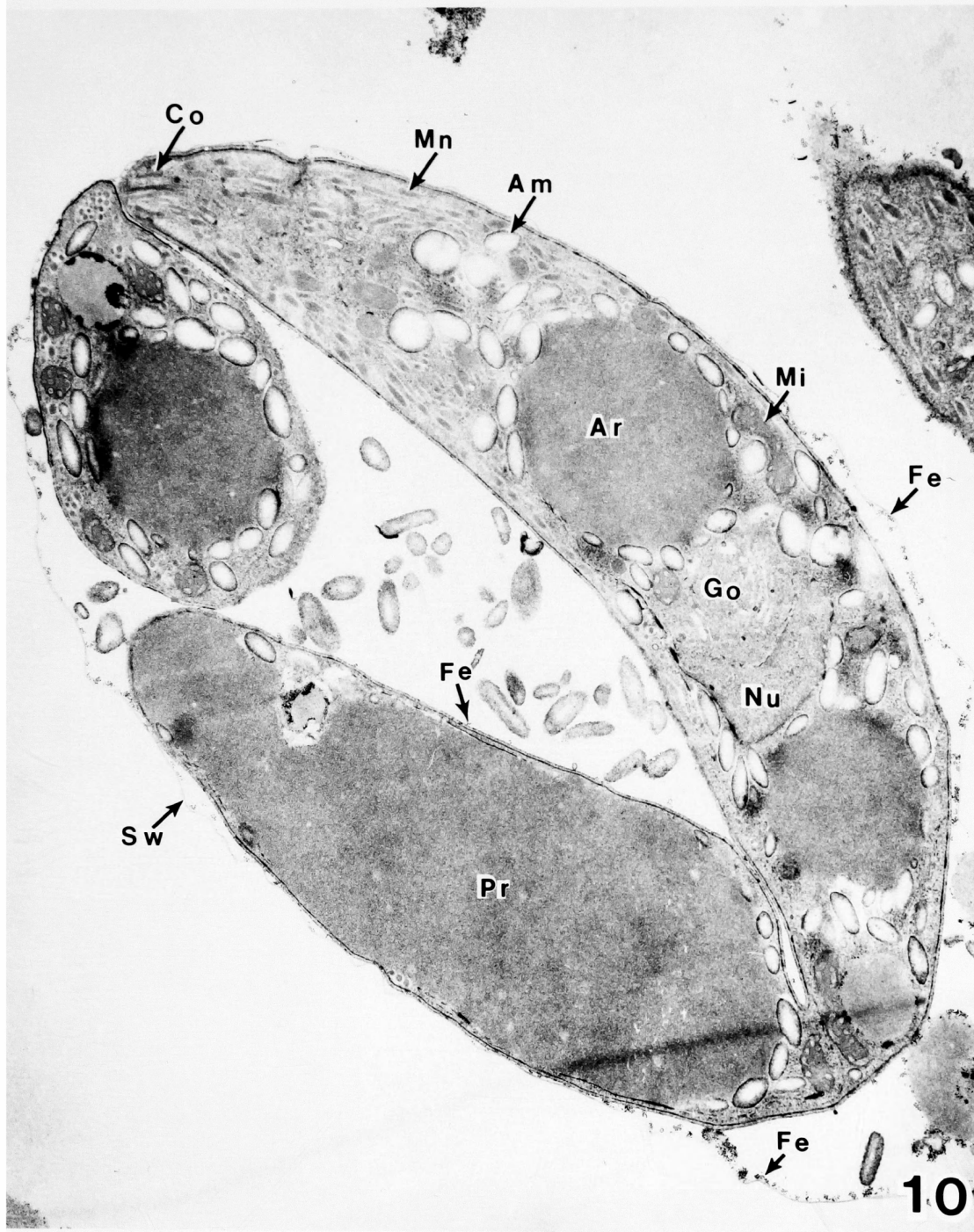


Plate 5. Ultrastructural localization of parasite-specific IgG on surface of sporozoite.

Figure 11. Longitudinal section of a sporozoite; ferritin (Fe) is localized in clumps on the plasmalemma. Ar, anterior refractile body; Mi, mitochondrion; Nu, nucleus; Pr, posterior refractile body. Treatment: Prefixed, immune serum, ferritin-conjugated goat anti-mouse IgG. X 19,800.

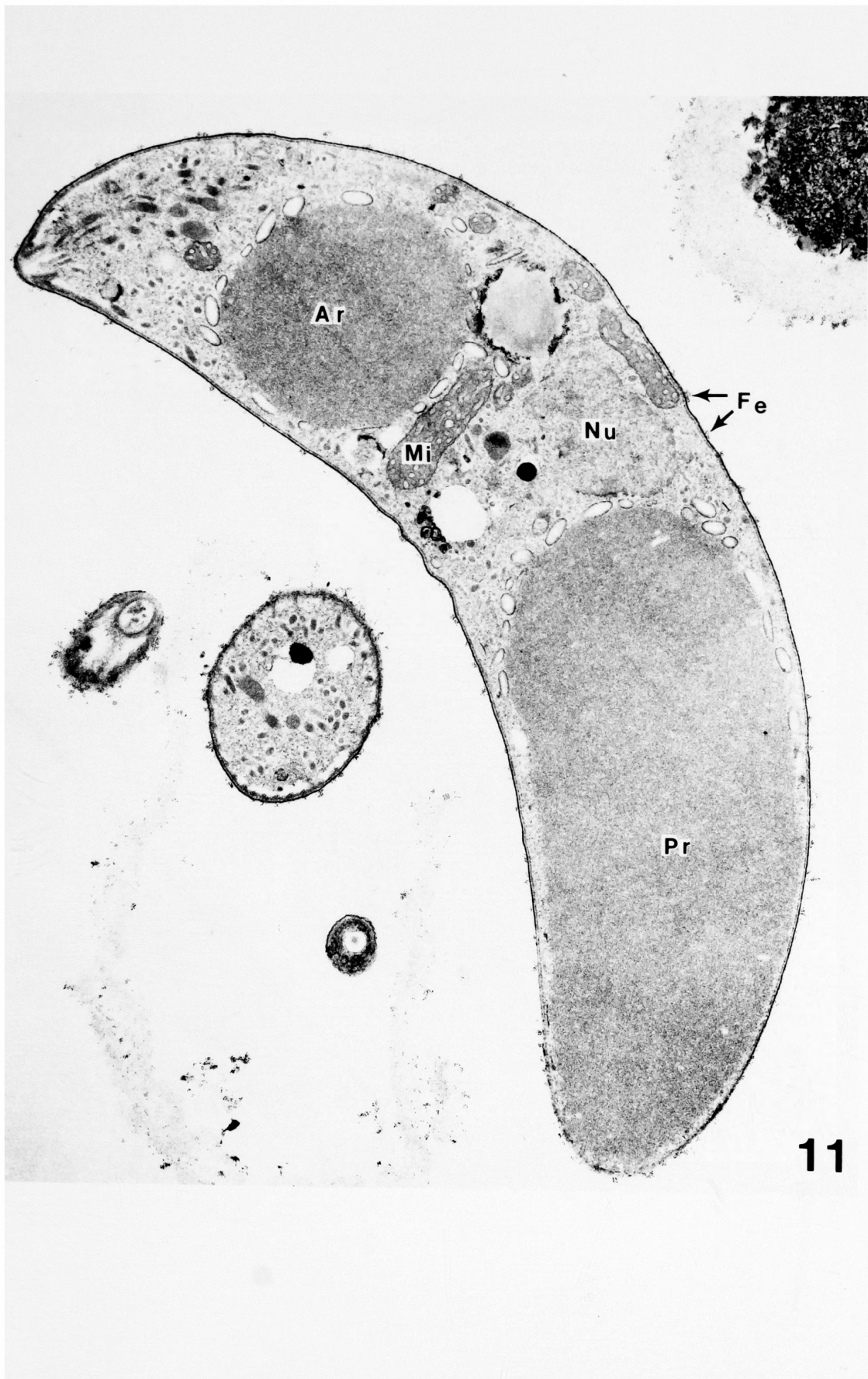


Plate 6. Ultrastructural localization of parasite-specific IgG on merozoites.

Figure 12. Third-generation merozoite with relatively uniform layer of ferritin (Fe). Co, conoid; Rh, rhoptry. Note flagellated protozoan (Fp) with no ferritin. Treatment: Prefixed, immune serum, ferritin-conjugated goat antimouse IgG. X 24,900.



Plate 7. Several merozoites exposed to normal serum and ferritin-conjugated goat antimouse IgG.

Figure 13. Third-generation merozoites. Note lack of ferritin on exposed surfaces. Treatment: Prefixed, normal serum, ferritin-conjugated antimouse IgG. X 10,890.

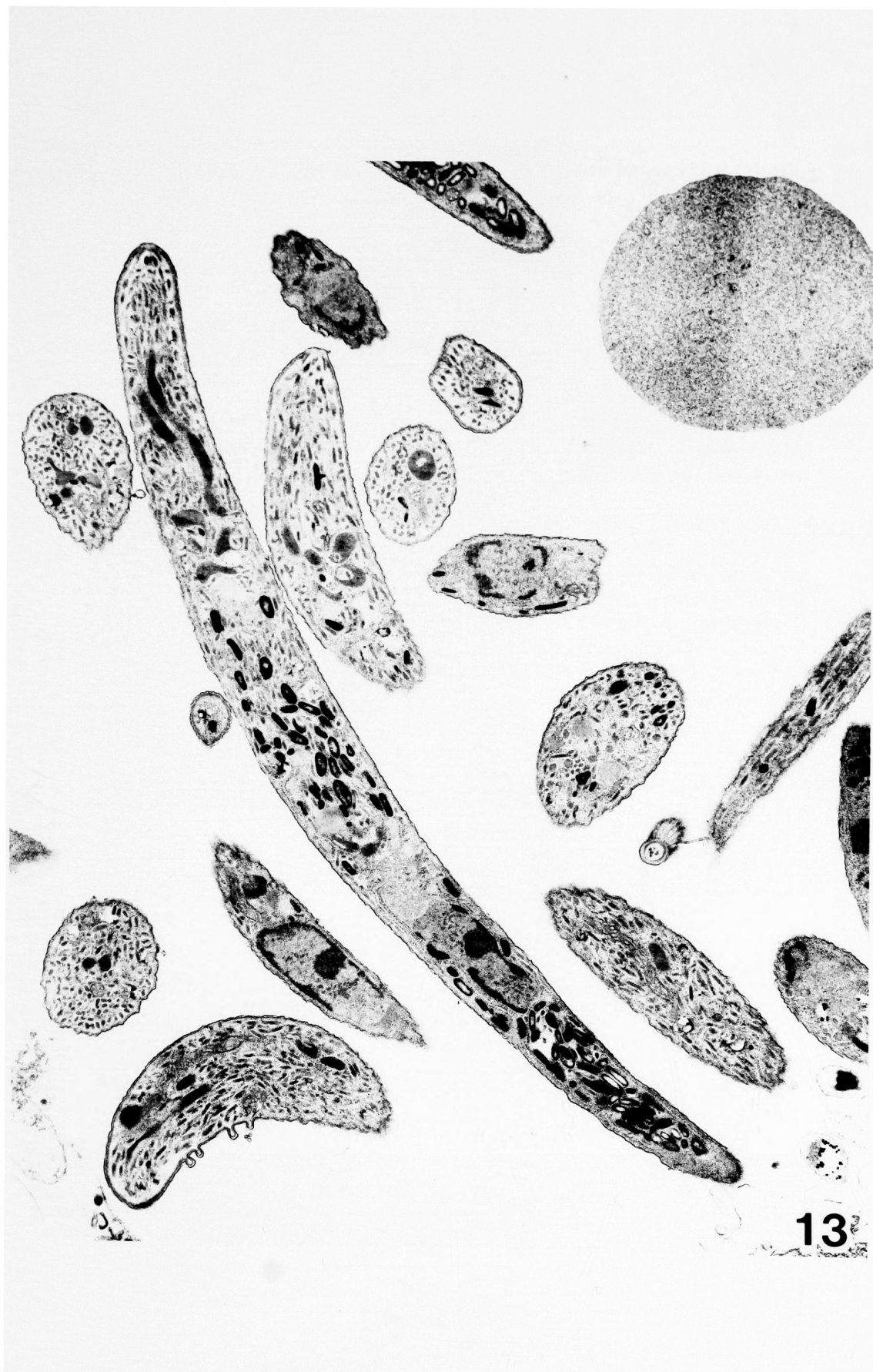
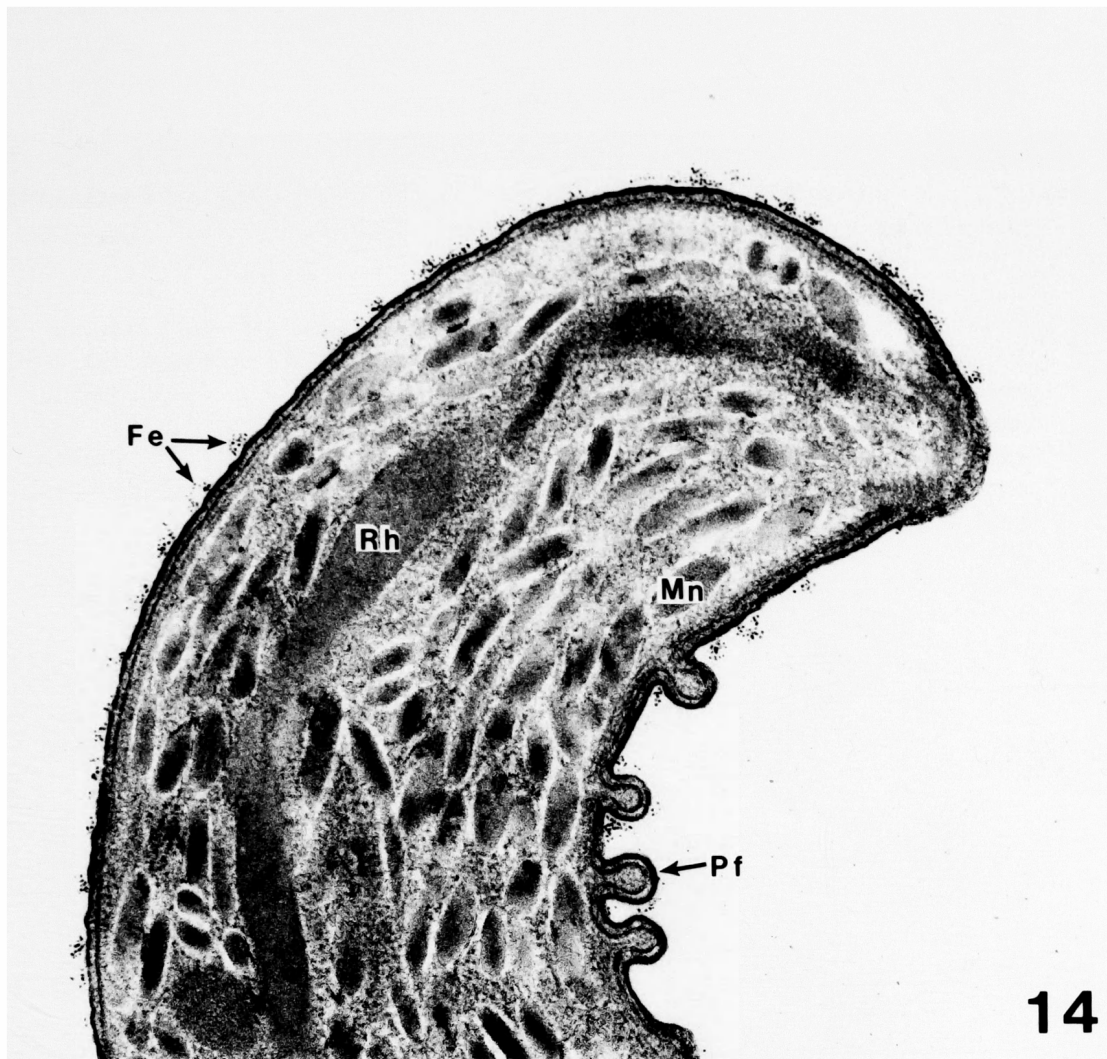


Plate 8. Ultrastructural localization of parasite-specific mouse IgG by ferritin-conjugated goat antimouse IgG on the surfaces of merozoites.

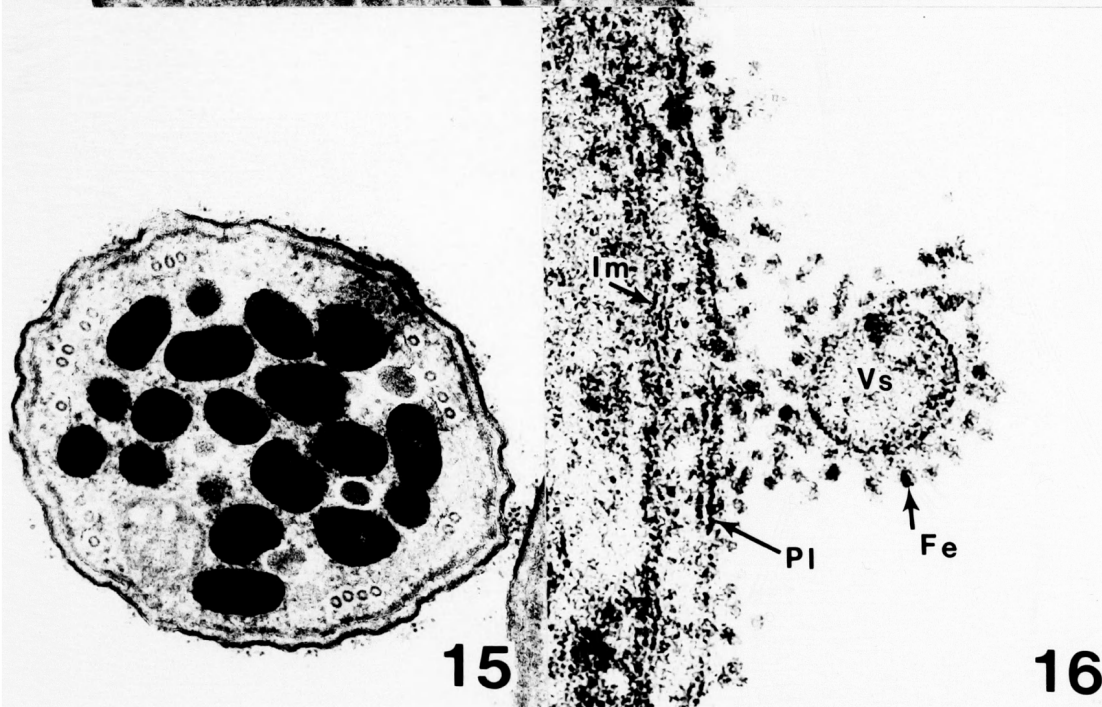
Figure 14. Anterior end of third-generation merozoite with clumps of ferritin (Fe) on its surface. Mn, microneme; Pf, pellicular fold; Rh, rhoptry. Treatment: Prefix-ed, immune serum, ferritin-conjugated goat antimouse IgG. X 54,000.

Figure 15. Cross-section of a merozoite near its posterior. Treatment: Prefix-ed, immune serum, ferritin-conjugat-ed goat antimouse IgG. X 62,500.

Figure 16. High magnification of pellicle of merozoite. Ferritin (Fe) has formed a relatively uniform layer on the merozoite plasmalemma (Pl) as well as on an ex-tracellular membrane-bound vesicle (Vs). Such vesi-cles may arise from the parasite plasmalemma. Im, inner membrane complex. Treatment: Prefix-ed, immune serum, ferritin-conjugated goat antimouse IgG. X 270,000.



14



15

16

Plate 9. Shedding of immune complexes from the plasmalemma near the posterior end of a merozoite.

Figure 17. High magnification of posterior of merozoite that was post-fixed after a 10 minute exposure to immune serum. Note membrane-bound vesicles (Vs) that were shed and ferritin attached to extracellular aggregates. Pl, plasmalemma; Mt, microtubule. Treatment: Post-fixed, immune serum, ferritin-conjugated goat antimouse IgG. X 99,000.

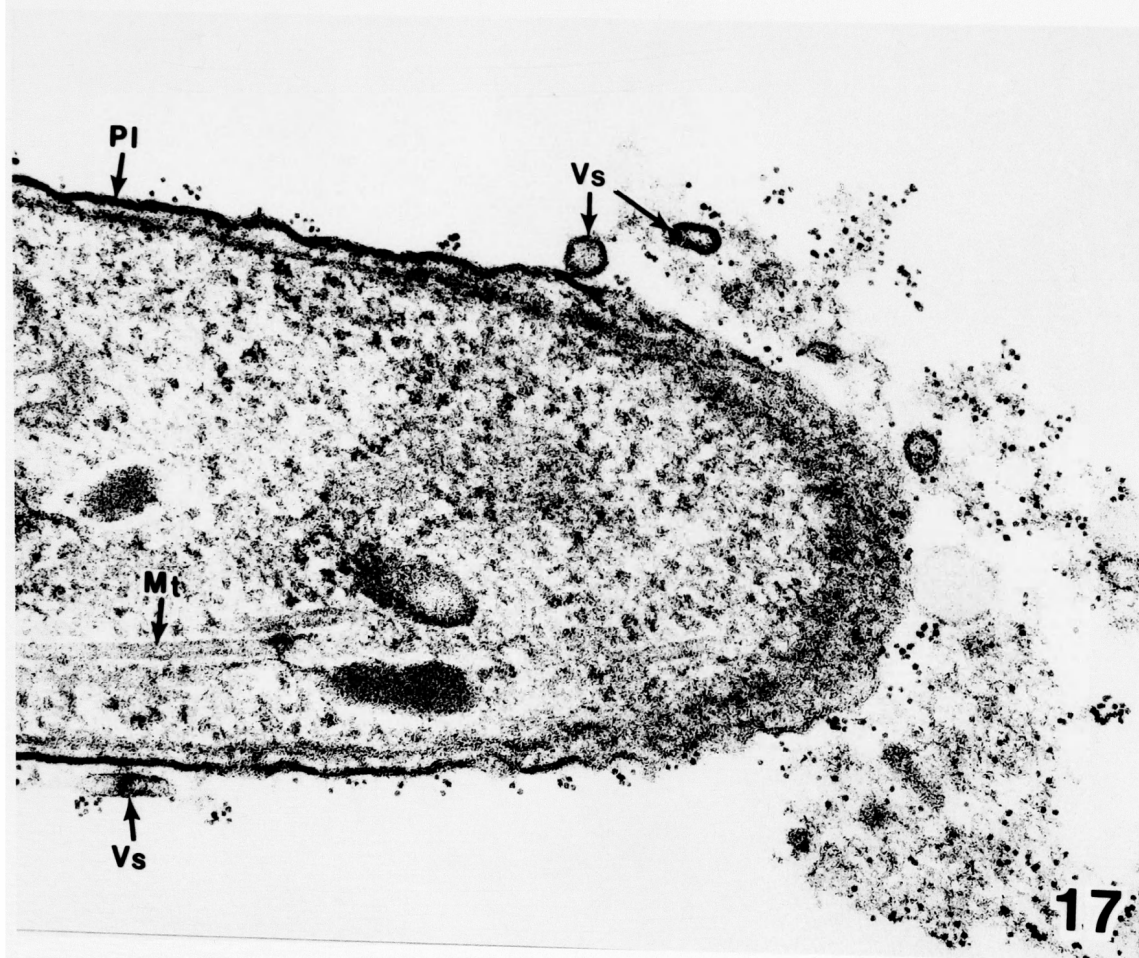


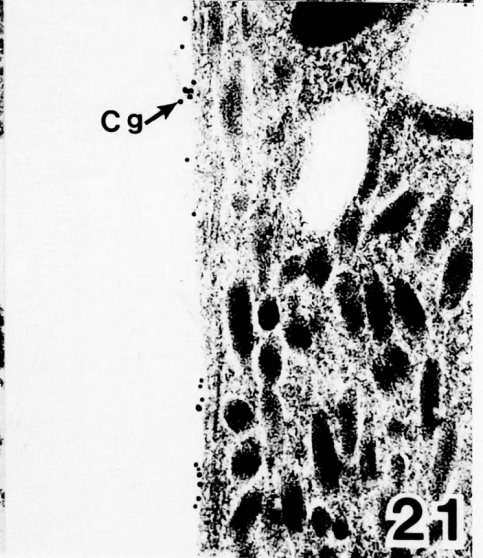
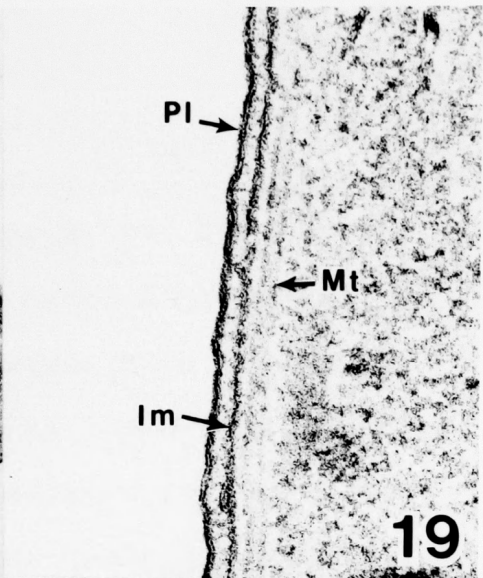
Plate 10. Shedding of ferritin labeled IgG immune complexes and ultrastructural localization of parasite-specific IgA on sporozoites and merozoites.

Figure 18. Merozoites which have shed most of their immune complexed outer membrane after a 15 minute exposure to immune serum. Note the small amounts of ferritin attached to merozoite plasmalemma and the relatively high concentrations of ferritin associated with extracellular membrane material (double arrows). Treatment: Post-fixed, immune serum, ferritin-conjugated goat antimouse IgG. X 21,120.

Figure 19. High magnification of pellicle of merozoite. Note lack of labeling of plasmalemma (Pl). Im, inner membrane complex; Mt, microtubule. Treatment: Prefixed, immune cecal contents, colloidal gold-conjugated goat antimouse IgA. X 115,500.

Figure 20. Surface of sporozoite labeled with colloidal gold (Cg). Treatment: Prefixed, immune cecal contents, colloidal gold-conjugated goat antimouse IgA. X 91,000.

Figure 21. Surface of merozoite labeled with colloidal gold (Cg). Treatment: Prefixed, immune cecal contents, colloidal gold-conjugated goat antimouse IgA. X 56,000.



DISCUSSION

Even though several species of Eimeria have been found to be fairly immunogenic (58), specific humoral and cell-mediated immune responses to Eimeria species have yet to be fully characterized. Work with phagocytes and T- and B-cell deficient animals indicates that cell-mediated immunity, particularly T-cells, is more important than immunoglobulins, yet antibodies still appear to play an important role (50, 54-55).

Specific immunoglobulins have been shown to modify Eimeria infections (46, 48). For example, transfer of immune serum to normal recipients affords some protection against the merogenous stages, but little or no protection during the sexual stages of the infection (47). This would seem reasonable since the extracellular forms of the parasite, mainly the sporozoites and merozoites, would be the most likely forms to come into contact with parasite-specific antibodies. Parasite-specific IgG can cause complement-mediated lysis as well as immobilization and opsonization of sporozoites and merozoites (48, 50, 52). Secretory IgA may also play a role in immune responses to infection with Eimeria species. Davis et al. (11), discovered that the numbers of IgA-containing cells in the ceca of chickens increased as well as the IgA concentration of cecal contents in response to infection with E. tenella. Additionally, Douglass

and Speer (14) found that sporozoites of E. falciformis exposed to enterocyte-associated mucus from immune mice had significantly shorter length/width ratios than sporozoites exposed to normal enterocyte-associated mucus. They attributed the difference in length/width ratios to the parasite-specific secretory IgA present in the mucus of immune animals (14).

Little or no information exists on the role of IgE and IgM in immunity to Eimeria species. Human IgM antibodies appear to opsonize and lyse zoites of Toxoplasma gondii (70).

In the present study, the oocyst-output of mice inoculated with sporozoites or merozoites of E. falciformis that had been previously exposed to heat-inactivated immune serum was 64.5% and 63.2% fewer than that of control mice. These results are similar to those of Rose (51) who found that E. maxima sporozoites incubated with heat-inactivated immune chicken serum caused a 46.1% decrease in oocyst-output when inoculated into naive birds. However, Rose (51) noted that sporozoites treated with normal serum resulted in an increase in oocyst-output. In contrast, I found a 19.4% and 18.4% decrease in oocyst-output by mice inoculated intracecally with sporozoites or merozoites, respectively, that had been treated previously with normal mouse serum. Thus, normal mouse serum appeared to have a slight deleterious effect upon sporozoites and merozoites of E. falciformis, whereas immune serum had a much greater inhibitory effect. I at-

tempted no in vivo inoculations of sporozoites or merozoites treated with immune cecal contents because only minimal titers of parasite-specific IgA were discovered in these contents by either IFA or IEM.

Davis et al. (10) found that cecal contents from immune chickens interfered with the ability of sporozoites of E. tenella to penetrate and develop in chick kidney cells in vitro. Cecal contents, regardless of immunoglobulin content or sporozoite neutralizing activity, were also found to impair in vitro development of the parasite (10). Based upon these findings, Davis et al. (10) suggested that parasite-specific secretory IgA plus proteolytic enzymes which are also present in the cecum may interfere with the ability of parasites to penetrate host cells. Parasites that penetrated host cells may have been sufficiently altered so that they were incapable of undergoing further development (10).

In the present study, IgG antibodies in the serum from specifically immunized mice were found to react with the inner and outer layers of the oocyst wall, inner surface of the sporocyst wall and the plasmalemma of sporozoites and merozoites of E. falciformis. This indicates that all of these parasite structures contain common or similar antigens. However, parasite-specific IgA antibodies from immune cecal contents were found to react only with the plasmalemma of sporozoites and merozoites. This indicates that there are also differences in the antigenic makeup among various

parasite structures or stages.

There were no apparent ultrastructural changes in sporozoites and merozoites of E. falciformis after exposure to heat-inactivated immune serum. Speer et al. (63) found no pellicular alterations of E. tenella sporozoites after exposing them to high titers of heat-inactivated monoclonal IgG antibodies. However, exposure to E. tenella sporozoites to nonheat-inactivated monoclonal immunoglobulins or immune serum resulted in complement-mediated pellicular damage and lysis of these organisms (63, 71).

Although no alterations in sporozoites and merozoites of E. falciformis were detected by TEM, immune serum exposure did have a deleterious effect on at least some of the sporozoites and merozoites because of their diminished ability to complete development in vivo.

Detection of parasite-specific antibody by IFA correlated well with that of IEM. However, IEM detected specific IgG bound to the inner surface of the sporocyst wall whereas IFA did not. Speer et al. (63) found that monoclonal IgG immunoglobulins were localized in a relatively uniform layer on a membranous covering of sporocyst walls and Stieda bodies of E. tenella. Differences in localization of antibody receptors on sporocysts of E. falciformis and E. tenella are probably due to differences in sporocyst development which results in structural differences as indicated by unit membrane locations within sporocysts. Sporocysts of

E. falciformis are lined internally but not externally by a unit membrane. In contrast, E. tenella sporocysts are covered externally by a unit membrane. However, it is possible that hybridoma technology may result in high titers of specific antibodies directed against an epitope that may not be normally presented to the immune system of a host during a natural infection or route of infection.

Low titers of parasite-specific IgA in immune cecal contents were also detected by IEM but seldom by IFA. Thus, because of the greater resolution of the electron microscope, the IEM system is more sensitive than IFA. Also, IEM appeared to be highly specific since I found no label on parasites or parasite structures that had been exposed previously to normal serum or cecal contents or labeled antibodies only. Furthermore, intestinal flagellates that were sometimes present in merozoite preparations exhibited no non-specific labeling after exposure to immune serum or cecal contents.

Relatively more ferritin labeled IgG occurred on the plasmalemma of merozoites than on sporozoites (compare Figs. 11-12, 14-16). This indicates that merozoites have relatively more of a particular antigen or more antigens in their plasmalemma than do sporozoites. This may be one of the reasons that merozoites of Eimeria species have been found to be more immunogenic than sporozoites (58). Merozoites are also much more numerous during an infection than are

sporozoites which may also enhance their antigenicity.

The ability of E. falciformis sporozoites and merozoites to cap and shed immune complexes from their plasmalemma may represent an evasive response of the parasite to host humoral or enteric antibody production. This may account for the inability of parasite-specific antibody to completely abolish parasite development in this present study. Since sporozoites and merozoites use their anterior ends to penetrate host cells, posterior capping and shedding of immune complexes may enable parasites exposed to parasite-specific antibodies to complete host cell penetration and undergo intracellular development. However, as suggested by Davis and Porter (56), antibodies or enzymes within digestive tracts of immune animals may not completely abolish host cell penetration, but may alter parasites rendering them incapable of undergoing intracellular development.

Capping of immune complexes has also been described for mammalian cells and various protozoan parasites such as Trypanosoma species, Leishmania species and Toxoplasma gondii (3, 7, 15-17, 21, 25, 65). Zoites of T. gondii appear to cap and shed immune complexes at their anterior end (5). In contrast, sporozoites and merozoites of E. falciformis (present study) and E. tenella (Speer et al., 64) cap and shed immune complexes posteriorly. Speer et al. (64) found that incubation in a monoclonal antibody alone did not by itself induce capping of immune complexes by

E. tenella sporozoites. More extensive cross-linkage, mediated by the second antibody ligand in the form of ferritin- or colloidal gold-conjugated antibody, was required to induce capping of plasmalemma antigens (64). These immune complexes were found to move laterally and posteriorly on the plasmalemma and form a cap at the posterior of the parasite (64). In contrast, the multivalency of parasite-specific IgG in this work enabled sufficient cross-linkage of immune complexes on the parasite plasmalemma for capping to proceed without the addition of a second antibody ligand.

No capping or shedding of parasite-specific IgA immunoglobulins was found on E. falciformis sporozoites or merozoites in this study. This was probably because the small amounts of IgA were unable to form sufficient cross-linkage on the plasmalemma of the parasites. However, Douglass and Speer (14) reported IgA titers of 1/64 in enterocyte-associated mucus from immune animals and found that mucus from immune mice attached to the surface of E. falciformis sporozoites and was subsequently translocated (capped) to the posterior of the parasite. It is possible that the techniques utilized in the present study did not dissociate low titers of IgA from the intestinal mucus of immune mice and thus failed to detect appreciable amounts of parasite-specific IgA. If so, this problem of purification and detection of IgA immunoglobulins from intestinal contents could prove formidable, since any drastic application of a reducing

agent such as dithiothreitol may cleave the IgA immunoglobulins as well as the enterocyte-associated mucus.

SUMMARY

A 64.5% and 63.2% decrease in oocyst-output occurred in mice inoculated intracecally with Eimeria falciformis sporozoites or merozoites, respectively, that had been previously treated with immune serum or immune cecal contents. Normal serum also caused a slight decrease (19.4% and 18.4%, respectively, for sporozoites and merozoites) in oocyst-output. Receptors for parasite-specific IgG were found on the surfaces of the outer and inner layers of the oocyst wall, the inner surface of the sporocyst wall, and on the plasmalemma of sporozoites and merozoites. Thus, these parasite structures appear to contain similar antigens. Sporozoites and merozoites capped and shed IgG-containing immune complexes at their posterior ends. Relatively few receptors for parasite-specific IgA were found on the plasmalemma of sporozoites and merozoites. No IgA receptors were found on sporocysts or oocysts. No ultrastructural alterations were detected in sporozoites and merozoites treated with heat-inactivated immune serum or immune cecal contents.

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